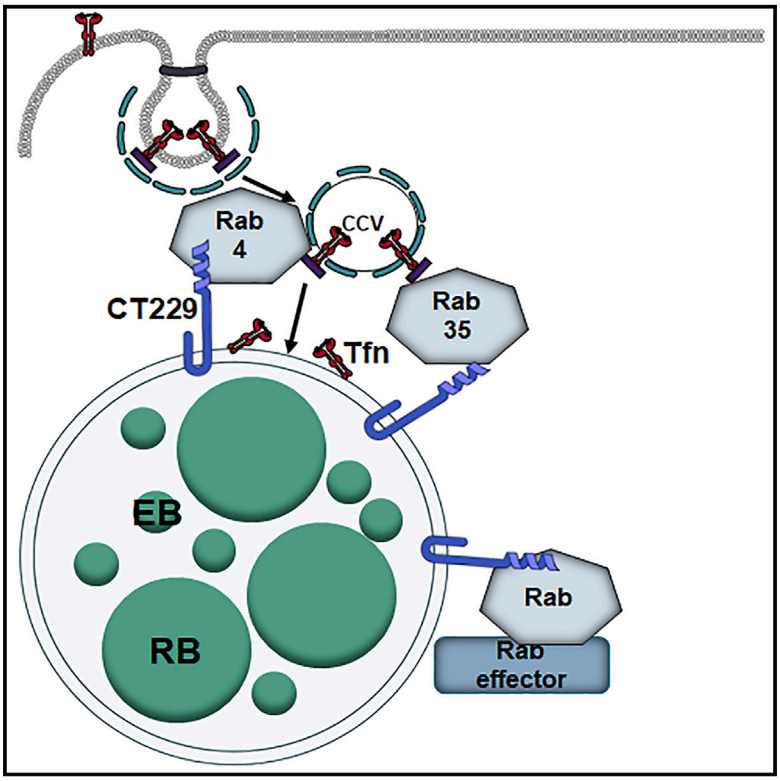
Article

Cell Reports

Chlamydia trachomatis CT229 Subverts Rab GTPase-Dependent CCV Trafficking Pathways to Promote Chlamydial Infection

Graphical Abstract



Authors

Robert Faris, Marlena Merling, Shelby E. Andersen, Cheryl A. Dooley, Ted Hackstadt, Mary M. Weber

Correspondence

mary-weber@uiowa.edu

In Brief

Faris et al. use genetic approaches to demonstrate that the disruption of the essential *Chlamydia trachomatis* Inc protein CT229 impairs recruitment of Rab GTPases, specific Rab effector proteins, transferrin, and the mannose-6-phosphate receptor to the periphery of the inclusion. Understanding how *C. trachomatis* hijacks host vesicular transport will help define how chlamydiae acquire essential substrates, such as iron and lipids, from the host cell.

Highlights

- CT229 recruits Rab GTPases and Rab effectors to the periphery of the inclusion
- The CT229 SNARE-like domain is necessary for binding and recruiting Rab GTPases
- CT229 and Rab GTPases are required to recruit Tfn to the periphery of the inclusion
- CT229 also recruits M6PR-containing vesicles to the vicinity of the inclusion

Faris et al., 2019, Cell Reports *26*, 3380–3390
March 19, 2019
https://doi.org/10.1016/j.celrep.2019.02.079

CellPress

OPEN ACCESS
CellPress

Cell Reports
Article

Chlamydia trachomatis CT229 Subverts Rab GTPase-Dependent CCV Trafficking Pathways to Promote Chlamydial Infection

Robert Faris,[1] Marlena Merling,[1,2] Shelby E. Andersen,[1] Cheryl A. Dooley,[3] Ted Hackstadt,[3] and Mary M. Weber[1,4,*]
[1]Department of Microbiology and Immunology, University of Iowa Carver College of Medicine, Iowa City, IA 52242, USA
[2]Department of Biology, University of Dayton, Dayton, OH 45469, USA
[3]Host Parasite Interactions Section, Laboratory of Bacteriology, Rocky Mountain Laboratories, National Institutes of Allergy and Infectious Disease, NIH, Hamilton, MT 59840, USA
[4]Lead Contact
*Correspondence: mary-weber@uiowa.edu
https://doi.org/10.1016/j.celrep.2019.02.079

SUMMARY

Chlamydial infection requires the formation of a membrane-bound vacuole, termed the inclusion, that undergoes extensive interactions with select host organelles. The importance of the Inc protein CT229 in the formation and maintenance of the chlamydial inclusion was recently highlighted by studies demonstrating that its absence during infection results in reduced bacterial replication, premature inclusion lysis, and host cell death. Previous reports have indicated that CT229 binds Rab GTPases; however, the physiological implications of this interaction are unknown. Here, we show that CT229 regulates host multivesicular trafficking by recruiting multiple Rab GTPases and their cognate effectors to the inclusion. We demonstrate that CT229 specifically modulates clathrin-coated vesicle trafficking and regulates the trafficking of transferrin and the mannose-6-phosphate receptor, both of which are crucial for proper chlamydial development. This study highlights CT229 as a master regulator of multiple host vesicular trafficking pathways essential for chlamydial infection.

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular pathogen responsible for a variety of serious human diseases. *C. trachomatis* serovars A–C are the leading cause of noncongential blindness worldwide (Satpathy et al., 2017), whereas serovars D–K are the etiological agents of one of the most common sexually transmitted infections (Menon et al., 2015). In women, 15%–40% of untreated infections can progress to pelvic inflammatory disease (PID), resulting in ectopic pregnancy and sterility (Menon et al., 2015). *C. trachomatis* serovars L1–L3 cause invasive urogenital or anorectal infections, known as lymphogranuloma venerum (LGV) (Stoner and Cohen, 2015). There is no vaccine, and, in total, over 100 million new *C. trachomatis* cases are reported annually (Menon et al., 2015). Furthermore, antibiotics fail to clear the infection in approximately 10% of cases (Afrakhteh et al., 2013; Geisler et al., 2015).

All chlamydiae share a biphasic developmental cycle in which they alternate between an infectious, environmentally stable elementary body (EB) and a noninfectious, replicative form termed the "reticulate body" (RB) (Elwell et al., 2016). During infection, the EB is internalized into a membrane-bound compartment, termed "the inclusion," that deviates from the endo-lysosomal pathway (Scidmore et al., 2003) and traffics along microtubules to the peri-Golgi region (Grieshaber et al., 2003). As obligate intracellular bacteria, chlamydiae must scavenge essential nutrients, such as amino acids, lipids, and iron from the host, all while avoiding detection by the host innate immune system (Elwell et al., 2016; Finethy and Coers, 2016; Pokorzynski et al., 2017). To achieve this, the chlamydial inclusion maintains intimate interactions with select host organelles, such as mitochondria (Chowdhury et al., 2017), the Golgi apparatus (Carabeo et al., 2003; Heuer et al., 2009), the endoplasmic reticulum (Derré et al., 2011), and endosomes (Saka et al., 2015). However, the precise mechanisms by which chlamydiae control these diverse interactions are largely unknown, but likely involve bacterial effector proteins capable of redirecting and intercepting host vesicles.

C. trachomatis encodes a type III secretion system (T3SS) that is predicted to translocate over 100 proteins into the host cell (Muschiol et al., 2011; Scidmore-Carlson et al., 1999; Subtil et al., 2005; Weber et al., 2015). A subset of proteins, termed inclusion membrane proteins (Incs), possess a bilobed hydrophobic domain of ~30–40 amino acids and are inserted in the inclusion membrane such that their N and C termini are oriented into the host cell cytosol (Hackstadt et al., 1999). Given their positioning at the host-pathogen interface, Incs likely mediate crucial interactions with the host cell. The importance of the Inc protein CT229 in forming and maintaining the unique intracellular niche of *C. trachomatis* is underscored by our recent study demonstrating that the absence of CT229 triggers premature inclusion lysis and host cell death (Weber et al., 2017). CT229 (CpoS) binds several Rab GTPases (Mirrashidi et al., 2015; Rzomp et al., 2006; Sixt et al., 2017). However, the physiological effects of CT229 targeting Rab GTPases and the necessity of this interaction for *C. trachomatis* infection is largely unknown.

To acquire key nutrients from the host and membrane for the growing inclusion, chlamydiae interact with and modulate aspects of intracellular trafficking and fusogenicity with the inclusion membrane. Movement and fusion of eukaryotic membrane bound vesicles is tightly regulated by soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors (SNARE) (Kim and Gadila, 2016), and small guanosine triphosphate (GTP) binding proteins, such as ADP-ribosylating factors (ARFs) (Donaldson and Jackson, 2011) and Rab GTPases (Hutagalung and Novick, 2011; Hammer and Wu, 2002). Rab GTPases localize to distinct organelles and regulate vesicle budding, transport, docking, and fusion (Hutagalung and Novick, 2011). Therefore, components of the eukaryotic vesicle trafficking apparatus are likely targeted by *C. trachomatis* effector proteins. Rab GTPases associated with both early endosomes (Rab4 and Rab11) and the Golgi apparatus (Rab1, Rab6, and Rab10) are recruited to the chlamydial inclusion membrane (Rzomp et al., 2003), suggesting that chlamydia diverts and interacts with Rab GTPases to hijack host vesicular trafficking.

Here, we show that CT229 recruits multiple Rab GTPases and Rab effector proteins to the inclusion; the CT229 Inc protein redirects and intercepts host clathrin-coated vesicles (CCVs). CT229 is required for recruitment of transferrin (Tfn) positive CCV's from the recycling pathway to the periphery of the inclusion and is required to redirect cation-independent mannose-6-phoshate receptor (CI-M6PR)-containing vesicles to the vicinity of the inclusion. These findings highlight CT229 as a potential master regulator of host CCV trafficking and provide the first conceptual insights as to how *C. trachomatis* subverts intracellular trafficking pathways to acquire essential nutrients.

RESULTS

CT229 Binds and Recruits Rab GTPases to the Inclusion during Infection

Large-scale screens using cells transfected with CT229 (Mirrashidi et al., 2015; Rzomp et al., 2006) or cells infected with *C. trachomatis* overexpressing CT229 (Sixt et al., 2017) suggest that CT229 binds multiple Rab GTPases. However, minimal overlap in Rab binding partners was noted between the studies. Immunoprecipitation of flag-tagged CT229 from transfected cells confirmed that CT229 does indeed bind each of the Rab GTPases previously reported (Figures 1A and S1A). Importantly, CT229 does not bind all Rab GTPases. Previous studies have demonstrated that Rab11 is recruited to the inclusion during infection (Rzomp et al., 2003). However, immunoprecipitation experiments revealed CT229 does not bind Rab11, demonstrating that CT229 does not indiscriminately bind to all Rab GTPases.

To exploit host vesicular trafficking pathways, many intracellular pathogens recruit or exclude specific Rab GTPases (Jennings et al., 2017; Qiu and Luo, 2017; Sherwood and Roy, 2013). Rab GTPases are recruited to the chlamydial inclusion in both a species-dependent and species-independent manner (Rzomp et al., 2003), suggesting that chlamydia modulates host vesicular trafficking by controlling Rab-dependent trafficking events. As shown in Figure 1B, Rab1, 4, 35, 8, 10, 11, 14, 34, and 6 localized in close proximity to the wild-type *C. trachomatis* L2 inclusion, whereas Rab18 and 33 did not. To determine whether CT229 expression is necessary for recruitment of these GTPases, we quantified Rab signal intensity at the inclusion in cells infected with wild-type, CT229::*bla*, or CT229::*bla* comp. Insertional inactivation of CT229 resulted in reduced Rab accumulation near the inclusion, except for Rab11 (Figures 1B and S1B), which was recruited independently of CT229 (Figure 1A). Complementation of the CT229::*bla* mutant with full-length CT229 restored Rab localization (Figures 1B and S1B). Immunofluorescence (IF) staining using Rab35, 11, or 8 antibodies confirmed endogenous Rab GTPases accumulate in close proximity to the chlamydial inclusion (Figure 1C), and this is dependent on CT229 (Figure S1C). Collectively, for the first time, our results show that Rab35, 8, 10, and 34 localize in close proximity to *C. trachomatis* L2 inclusions in a CT229-dependent manner.

Rab proteins cycle between an inactive GDP-bound and an active GTP-bound state. GTP binding induces a conformational change, exposing amino acid residues required for binding to Rab effector proteins (Weber and Faris, 2018). Because Rab GTPases interact with Rab effector proteins in a nucleotide-dependent manner and previous studies with Rab4 imply that CT229 preferentially binds the GTP form (Rzomp et al., 2006), we sought to determine whether CT229 interacts with the GTP-bound form of additional Rab GTPases. We focused on Rab1 as a representative GTPase that regulates post-Golgi trafficking and Rab4 and 35 as representative GTPases that regulate protein recycling from the plasma membrane (Weber and Faris, 2018). Reduced binding to the GDP-locked, dominant-negative Rab was observed (Figure S2A). In line with this observation, GDP-locked Rab GTPases were not observed in close proximity to the inclusion, whereas GTP-locked constitutively active or wild-type Rab proteins were (Figure S2B). Collectively, our results suggest that CT229 preferentially binds and recruits GTP-bound Rab GTPases to the vicinity of the chlamydial inclusion, potentially to promote downstream signaling events through interactions with Rab effector proteins.

CT229 Modulates Recruitment of Key Rab Effector Proteins to the Inclusion

Rab effector proteins preferentially interact with the GTP-bound form of the Rab GTPase and propagate downstream signals to promote a plethora of molecular interactions including vesicle budding, transport, tethering, and fusion (Hutagalung and Novick, 2011). Oculocerebrorenal syndrome of Lowe (OCRL) is an inositol-5 phosphatase that regulates clathrin-coated pit formation and regulates endosomal sorting and trafficking of CI-M6PR and transferrin through its interaction with Rab35 (Cauvin et al., 2016). OCRL also can act as an uncoating factor that facilitates recycling of endocytic factors (Nández et al., 2014). OCRL was previously shown to be recruited to the chlamydial inclusion and depletion of OCRL significantly reduced chlamydial replication (Moorhead et al., 2010). We hypothesized that recruitment of Rab effector proteins to the inclusion is due to the binding of CT229 with its cognate Rab GTPase and that the absence of CT229 would result in a failure to recruit Rab effectors to the inclusion. As previously reported, OCRL was observed in close proximity to wild-type inclusions (Figure 2), but not CT229::*bla*

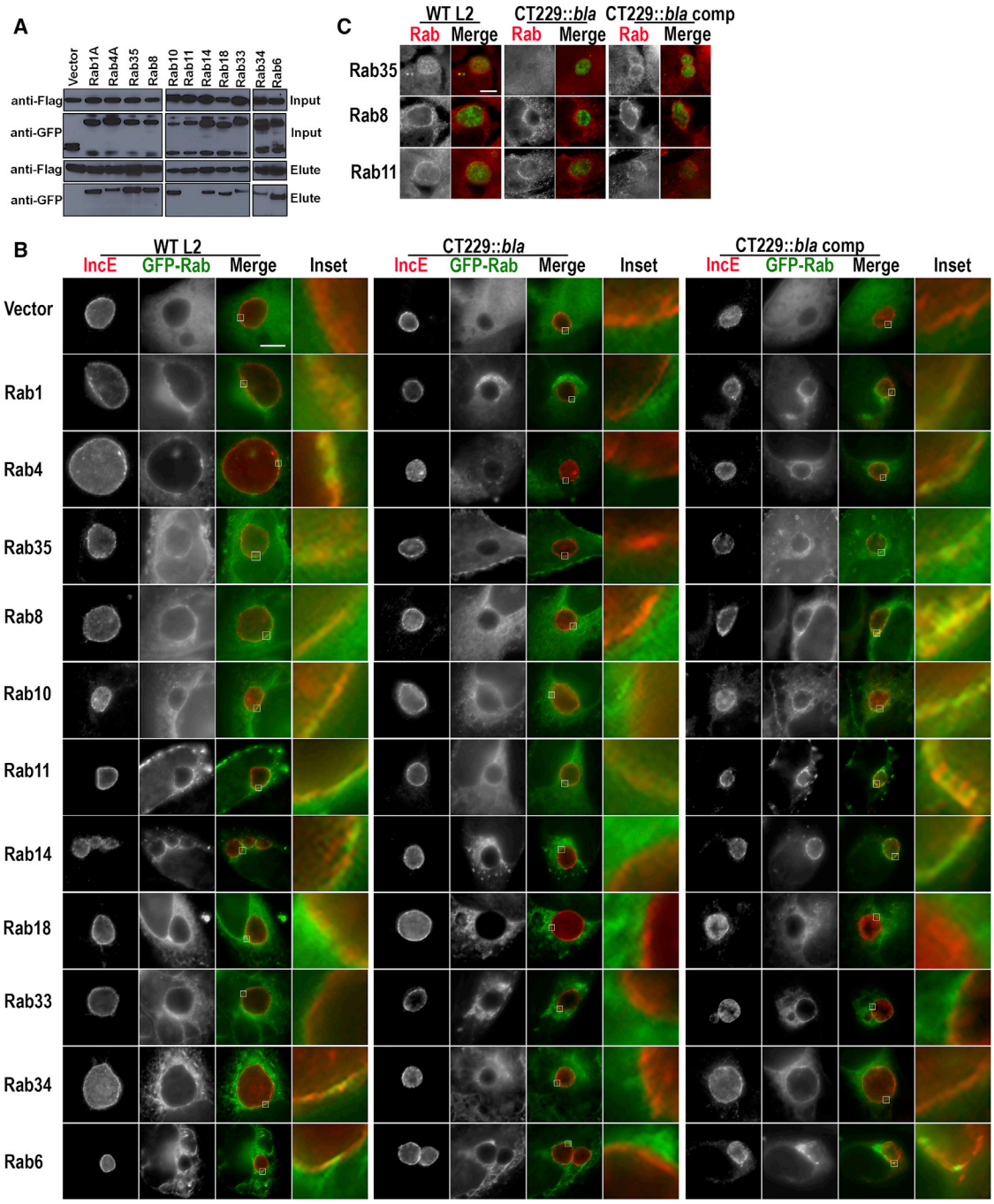


Figure 1. Recruitment of Multiple Rab GTPases to the Inclusion Is Dependent on the Inclusion Membrane Protein CT229

(A) HeLa cells were transfected with GFP-tagged Rab GTPases and infected with *C. trachomatis* L2 pBomb4-tet-CT229 flag at an MOI of 2.5. The flag-tagged fusion was immunoprecipitated, and samples were resolved by western blotting. Data are representative of three independent experiments.

(B) IF was conducted on HeLa cells transfected with Rab GTPases and infected at an MOI of 1 for 18 h. Cells were fixed at 18 h post-infection with formaldehyde and probed with an anti-IncE antibody (red). Scale bar represents 10 μm. White boxes denote area used for inset. Data are representative of three independent experiments.

(C) IF was conducted on HeLa cells infected at an MOI of 1. Cells were fixed at 18 h post-infection with formaldehyde or methanol and probed with an anti-Momp antibody (green) or anti-Rab35, 8, or 11 (red) antibodies. Scale bar represents 5 μm.

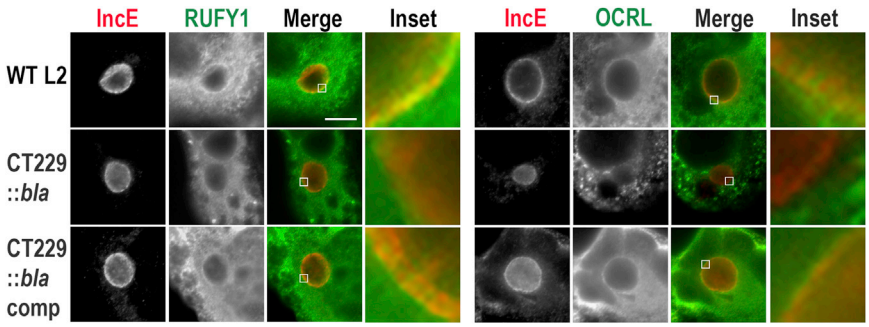


Figure 2. Recruitment of Rab Effector Proteins to the Chlamydial Inclusion Requires CT229

HeLa cells were transfected with HA-OCRL or GFP-RUFY1 and infected at an MOI of 1 for 18 h. Cells were fixed with formaldehyde or methanol and probed with anti-IncE (red) and anti-HA (OCRL) antibodies. Scale bar represents 10 μm. Data are representative of three independent experiments.

inclusions (Figure 2). Complementation of CT229::*bla* restored OCRL localization, demonstrating that recruitment of specific Rab effector proteins is influenced by CT229.

To determine whether Rab effector recruitment is specific to OCRL or whether recruitment of additional Rab effector proteins is influenced by CT229, we assessed recruitment of RUFY1, a Rab4 effector protein that is necessary for efficient transferrin recycling (Yamamoto et al., 2010). As shown in Figure 2, RUFY1 was observed in close proximity to the inclusion, and this localization occurred in in a CT229-dependent manner. Thus, our results imply that CT229, through interactions with Rab GTPases, influences the recruitment of multiple Rab effector proteins.

Rab GTPases Are Essential for Normal Chlamydial Replication

The necessity of key Rab GTPases during chlamydial infection was further evaluated by reducing their expression in host cells. HeLa cells were transfected with small interfering RNAs (siRNAs) against Rab4, 35, and a non-targeting control. Additionally, Rab5 was included as a GTPase not involved in chlamydia infection, and Rab11 was included as a GTPase that is recruited to the inclusion independently of CT229. Knockdown (KD) was verified by western blotting (Figure S2C). Knockdown of Rab4 or 35 resulted in a reduction of infectious progeny compared to those cells treated with a non-targeting control (Figure S2C). Importantly, siRNA KD of Rab5 or 11 did not significantly reduce chlamydia infection compared to scrambled treated. These results imply that specific Rab GTPases are essential for chlamydial replication.

The Coiled-Coil SNARE-like Domain of CT229 Is Important for Mediating Binding and Recruitment of Rab GTPases

To address the role of CT229 in mediating host-pathogen interactions, we analyzed the coding sequence to identify putative functional domains potentially involved in co-opting the host. Similar to IncA (Delevoye et al., 2008), CT229 possesses a coiled-coil "SNARE-like domain" (SLD) comprising a heptad repeat sequence of hydrophobic residues with a conserved glutamine residue defining the zero layer (Figure 3A). CT229 also encodes a tyrosine-based sorting signal, YXXΦ (Φ-bulky hydrophobic amino acid) that in eukaryotic proteins binds the heterotetrameric clathrin adaptor protein (AP) complexes AP1, AP2, or AP3 (Robinson, 2015). Previous studies suggested that the C-terminal 18 residues (197–215) of CT229 are necessary but not sufficient for interaction with Rab4 in yeast (Rzomp et al., 2006).

As shown in Figures 3B and 4, CT229 expression in yeast impairs growth, suggesting that it targets an essential pathway. Experiments in yeast expressing CT229 variants further revealed the importance of the SLD in CT229 effector function, as a single amino acid substitution in this region rescued the growth defect while expression of $CT229^{\Delta 197\text{-}215}$ or $CT229^{Y116F}$ resulted in diminished growth similar to $CT229^{FL}$ (Figure 3B). Importantly, $CT229^{FL}$ and $CT229^{Y120D}$ were expressed at similar levels (Figure S3) indicating that the rescue of growth is not due to reduced protein expression. These results suggest that the coiled-coil SLD is necessary for CT229 effector function.

To directly test whether any of these functional domains are required for binding Rab GTPases during infection, we expressed $CT229^{FL}$, $CT229^{\Delta 197\text{-}215}$, or $CT229^{L120D}$ in wild-type *C. trachomatis*. Immunoprecipitation of Flag-tagged CT229 revealed that both $CT229^{\Delta 197\text{-}215}$ and $CT229^{L120D}$ exhibited reduced binding to Rab4 (Figure 3C). Thus, our results demonstrate that both the coiled-coil SNARE-like domain and the C terminus are necessary for binding Rab GTPases and carrying out CT229 effector function.

To confirm the importance of the SLD in CT229-dependent Rab recruitment, we expressed CT229 L120D, under the control of its native promoter, in CT229::*bla*. Growth curves in HeLa cells demonstrated that while expression of full-length CT229 in CT229::*bla* completely restored inclusion development and intracellular replication to wild-type levels, expression of the L120D variant resulted in only a slight rescue of intracellular replication (Figures 3D and 3E). To evaluate the role of the CT229 SLD in modulating Rab vesicle recruitment, we assessed the localization of Rab1 as a representative GTPase that regulates post-Golgi trafficking and Rab4 and 35 as representative GTPases that regulate protein recycling from the plasma membrane. Importantly, CT229::*bla* L120D comp was unable to recruit Rab GTPases to the vicinity of the inclusion (Figure 2F). Collectively, our results highlight the importance of the CT229 SLD in binding and recruiting Rab GTPases to the inclusion.

CT229 Targets Clathrin-Dependent Vesicle Transport Pathways

The yeast suppressor screen is an innovative technique that we (Weber et al., 2016a) and others (Guo et al., 2014; Tan and Luo, 2011; Tan et al., 2011) have used to identify host pathways targeted by bacterial effector proteins. Many bacterial effector

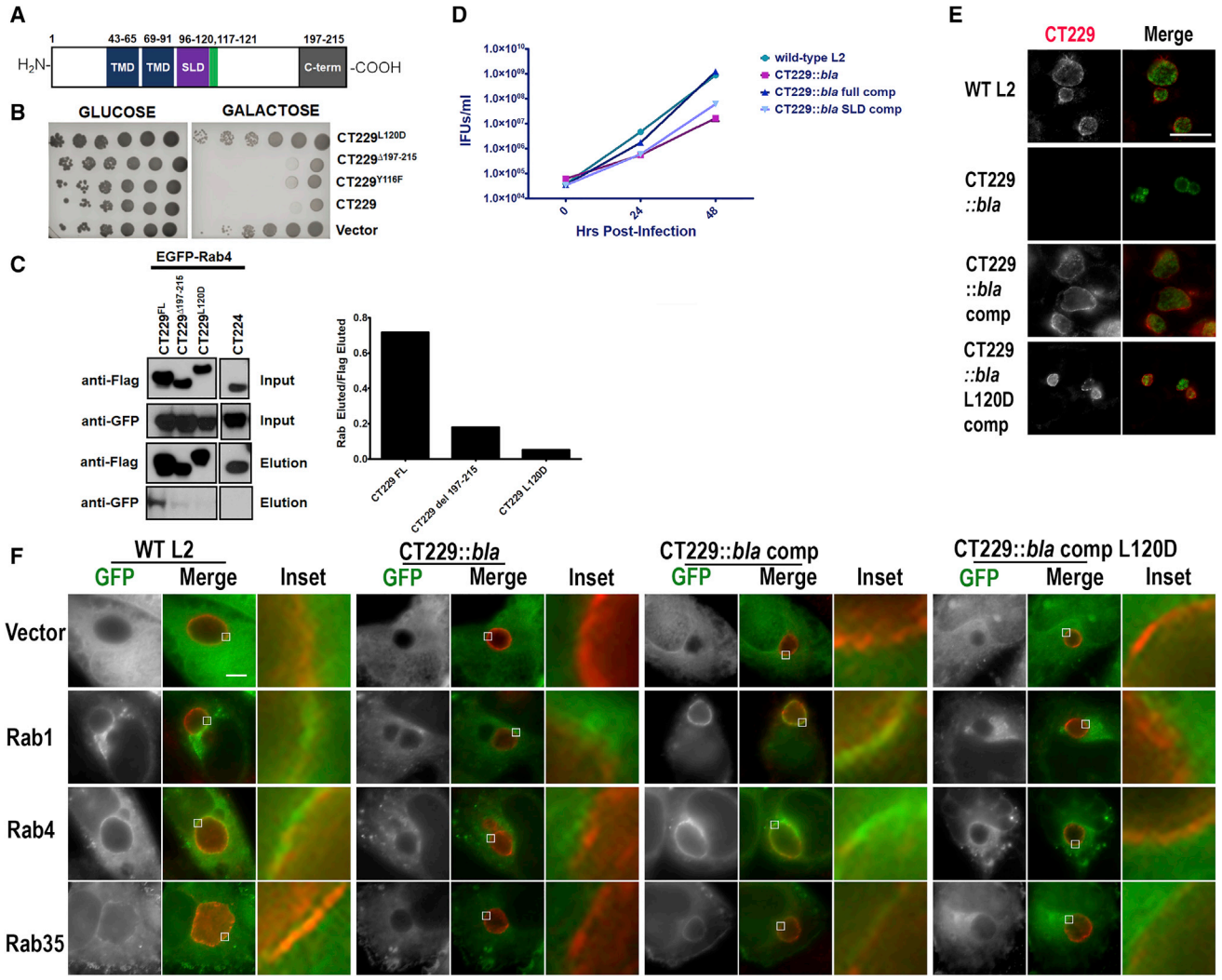


Figure 3. Coiled-Coil SNARE-like Domain of CT229 Is Necessary for Chlamydia Replication and Rab Recruitment

(A) Bioinformatic analysis of CT229 identified a transmembrane domain (blue), a coiled-coil SLD (purple), and Y-based sorting signal (green).

(B) QuikChange mutagenesis was used to determine whether any of the predicted motifs are necessary for CT229 toxicity in yeast. CT229 variants were transformed into *S. cerevisiae* W303 and spotted onto uracil dropout media to assess toxicity.

(C) To determine whether CT229 eukaryotic-like domains or the C terminus are required for CT229 binding to Rab GTPases, we introduced a single amino acid substitution into the SLD ($CT229^{L120D}$). $CT229^{FL}$ (full-length), $CT229^{\Delta 197-215}$, and $CT229^{L120D}$ were overexpressed as Flag-tagged fusions in *C. trachomatis*. Flag-tagged CT229 was immunoprecipitated from HeLa cells transfected with Rab4 and samples were analyzed by western blotting. Data are representative of three independent experiments.

(D) HeLa cells were infected at a MOI of 1; at 0, 24, and 48 h post-infection host cells were lysed; and infectious EBs were plated on new HeLa monolayers. Infectious-forming units (IFUs) were enumerated using IF microscopy. Data are representative of two independent experiments.

(E) HeLa cells were infected at a MOI of 2, and, at 24 h, post-infection cells were fixed and stained using anti-MOMP (green) and anti-CT229 (red) antibodies. Scale bar represents 20 μm. Data are representative of two independent experiments.

(F) IF was conducted on HeLa cells transfected with Rab GTPases and infected at an MOI of 1 for 18 h. Cells were fixed at 18 h post-infection with formaldehyde and probed with an anti-IncE antibody (red). Scale bar represents 10 μm. White boxes denote area used for inset. Data are representative of three independent experiments.

proteins modulate essential cellular signaling pathways (apoptosis, cytoskeletal function, vesicular traffic, etc.) and when overexpressed in yeast, effector targeting of these pathways manifests as impaired growth. This phenotype can be exploited to identify the cognate host pathways that are targeted by these effector proteins by conversely overexpressing a library of host proteins to identify those that restore yeast growth. To identify the yeast pathway targeted, we co-expressed full-length CT229 with a yeast genomic library. We identified 74 colonies in which the growth defect was rescued; however, only 12 of these consistently suppressed the growth defected associated with expression of CT229. Sequencing of suppressor plasmids

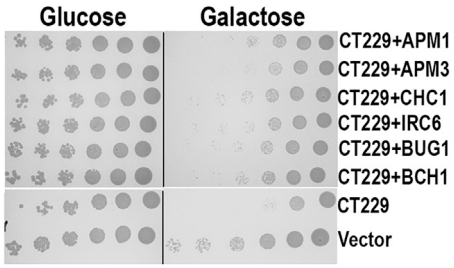


Figure 4. CT229 Perturbs Clathrin-Dependent Transport Pathways
S. cerevisiae W303 pYesNTA-CT229 was transformed with the yeast genomic library pYEp13. Twelve clones were isolated that consistently suppressed the toxicity of CT229.

revealed that all 12 clones contained open reading frames (ORFs) associated with components of vesicle trafficking pathways (BUG1, BCH1), components of the adaptor protein complex (APM1, APM3), clathrin (CHC1), or clathrin accessory factors (IRC6) (Figure 4). These results imply that CT229 targets clathrin-dependent vesicle transport pathways.

Key Host Vesicular Trafficking Pathways Are Modulated during *C. trachomatis* Infection in a CT229-Dependent Manner

Clathrin-coated vesicles (CCVs) are generated at the plasma membrane or *trans*-Golgi network (TGN) and facilitate the movement of cargo to endosomes (Weber et al., 2016b). Rab GTPases, such as Rab4 and Rab35, are key participants in CCV trafficking and facilitate docking, tethering, and fusion of CCVs with target membranes. Given that CT229 binds and recruits Rab vesicles to the inclusion and that CT229 toxicity in yeast could be suppressed by overexpressing clathrin, we sought to determine whether CT229 perturbs trafficking of key clathrin-dependent trafficking pathways. The TfnR is a cell-surface receptor that binds to transferrin (Tfn) in its iron bound form (holo-Tfn). Binding of holo-Tfn to the TfnR results in internalization of the TfnR:holo-Tfn complex via clathrin-dependent endocytosis where iron is released into a sorting endosome (Mayle et al., 2012). The Tfn:TfnR complex is subsequently recycled back to the cell surface either directly from the sorting endosome (fast recycling) or via a recycling endosome (slow recycling) (Mayle et al., 2012). Rab4 and 35 regulate fast recycling of Tfn whereas Rab11 regulates slow recycling of Tfn (Hutagalung and Novick, 2011; Mayle et al., 2012). Because CT229 binds Rab4 and 35, but not 11 (Figure 1A), we sought to determine whether CT229 is involved in modulating Tfn recycling. As previously noted (van Ooij et al., 1997; Scidmore et al., 1996), fluorescently labeled Tfn was noted at the periphery of wild-type inclusions (Figure 5A). Notably, the amount of Tfn at the periphery of the inclusion increased over time and the amount of Tfn on the surface of infected cells was decreased. Intriguingly, Tfn was notably absent from the periphery of CT229::*bla* inclusions, a phenotype that was rescued by complementation with full-length CT229 (Figure 5A). To better observe the localization of Tfn in chlamydial infected cells, we used immunoelectron microscopy with Tfn-HRP. Consistent with previous studies (Scidmore et al., 1996), Tfn-HRP localized to vesicles or tubular elements in close proximity to the chlamydial inclusion (Figure 5B). Conversely, Tfn-HRP was notably absent from CT229::*bla* inclusions. These results demonstrate that CT229 is necessary for recruitment of Tfn to the periphery of the inclusion.

To determine whether CT229 specifically modulates Tfn recycling or more broadly regulates endocytic recycling, we monitored the trafficking of the epidermal growth factor receptor (EGFR), a signaling pathway that was previously shown to be important for *C. trachomatis* development (Patel et al., 2014). While co-localization of the chlamydial inclusion with EGF was noted, only a minor decrease in recruitment to the CT229::*bla* inclusion was observed (Figure 5B). Thus, both EGF and Tfn are recruited to the chlamydial inclusion; however, only Tfn recruitment depends on CT229. Whether a compensatory pathway or another Inc mediates EGF recruitment remains unknown.

To determine whether CT229 only regulates CCVs from the endocytic pathway or whether CCV trafficking from the TGN is modulated by CT229, we evaluated the localization of the M6PR. M6PR are found in the TGN, endosomes, and plasma membrane (Braulke and Bonifacino, 2009). In the TGN, M6PR recognizes mannose-6-phosphate residues on newly synthesized lysosomal proteins and the M6PR-ligand complex is packaged into CCVs in the TGN, where it then traffics to endosomes. As the pH drops, the ligand dissociates and the M6PR is recycled back to the TGN (Braulke and Bonifacino, 2009). Bidirectional trafficking between the TGN and endosomes is mediated by several Rab GTPases including Rab6 (Progida and Bakke, 2016). To determine whether CT229 modulates transport between the TGN and endosomes, we assessed localization of M6PR during chlamydial infection. As previously noted (van Ooij et al., 1997), M6PR localized in close proximity to the inclusion. Importantly M6PR staining in close proximity to the inclusion was notably absent from CT229::*bla*, suggesting CT229 plays a role in recruiting M6PR-containing vesicles to the vicinity of the inclusion. Complementation of CT229::*bla* restored localization of M6PR-containing vesicles to the vicinity of the inclusion. Taken together our results indicate that CT229 modulates clathrin-dependent transport pathways from the plasma membrane and TGN.

Rab4 and 35 Are Essential for the Recruitment of Tfn to the Inclusion

Rab GTPases are key regulators of various host vesicle trafficking pathways. Tfn recycling via the fast pathway is regulated by Rab4 and 35, both of which are interacting partners of CT229 (Figure 1A). To confirm that Tfn recruitment to the inclusion by CT229 is dependent on an interaction with these Rab GTPases, Rab4, 35, and 11 were knocked down (KD) using siRNA and KD was confirmed by western blotting (Figure 6). Knockdown of Rab4 or 35 resulted in a significant reduction in Tfn localization to the periphery of the chlamydial inclusion whereas no defect in Rab11 KD cells was noted (Figure 6). These results indicate that trafficking of Tfn to the periphery of the inclusion requires Rab4 and 35 and confirms the necessity of CT229 in mediating this process.

DISCUSSION

Like many obligate intracellular pathogens, chlamydiae possess a substantially reduced genome (1.04 Mb, 895 ORFs) that lacks

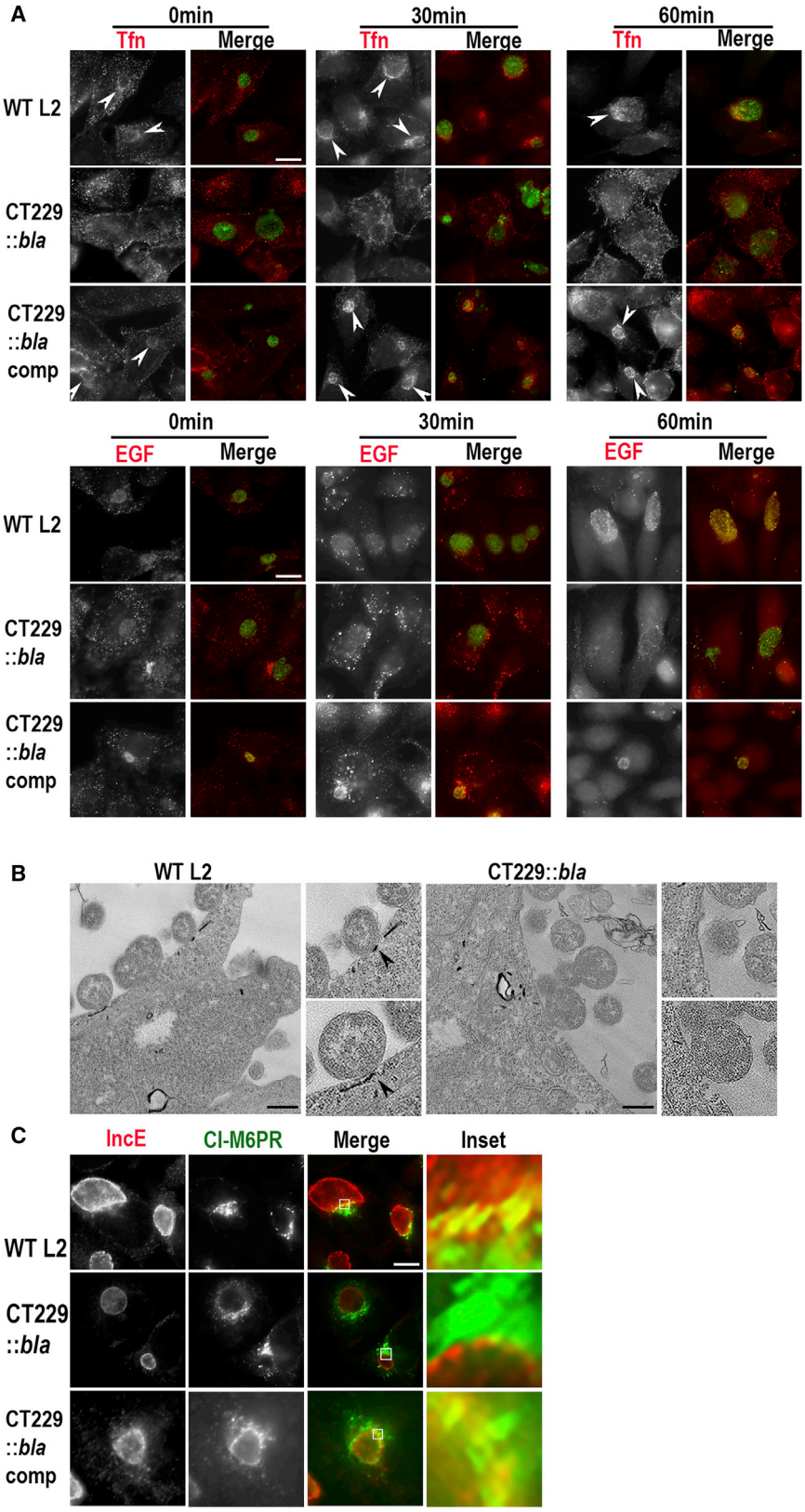


Figure 5. Tfn and CI-M6PR Recruitment to the Inclusion Requires CT229

(A) HeLa cells were infected for 18 h at an MOI of 1 and subsequently serum starved on ice for 30 min. Infected cells were loaded with Tfn AlexaFluor 594 or rhodamine EGF and incubated at 37°C. Cells were loaded with excess unlabeled Tfn or EGF for 0, 30, or 60 min and fixed with formaldehyde. Bacteria where stained with anti-L2 (green) antibodies. Data are representative of three independent experiments. White arrowheads indicate areas of recruitment. Scale bar represents 10 μm.

(B) Chlamydial-infected cells were incubated in the presence of Tfn-HRP and stained for electron microscopy using the immunoperoxidase method. Black arrowheads indicate areas of Tfn recruitment to the inclusion. Scale bars represent 500 nm.

(C) HeLa cells were infected at an MOI of 1 for 18 h and stained using an anti-M6PR (green) and anti-IncE (red) antibodies. White boxes denote the area used for inset. Scale bars represent 10 μm. Data are representative of three independent experiments.

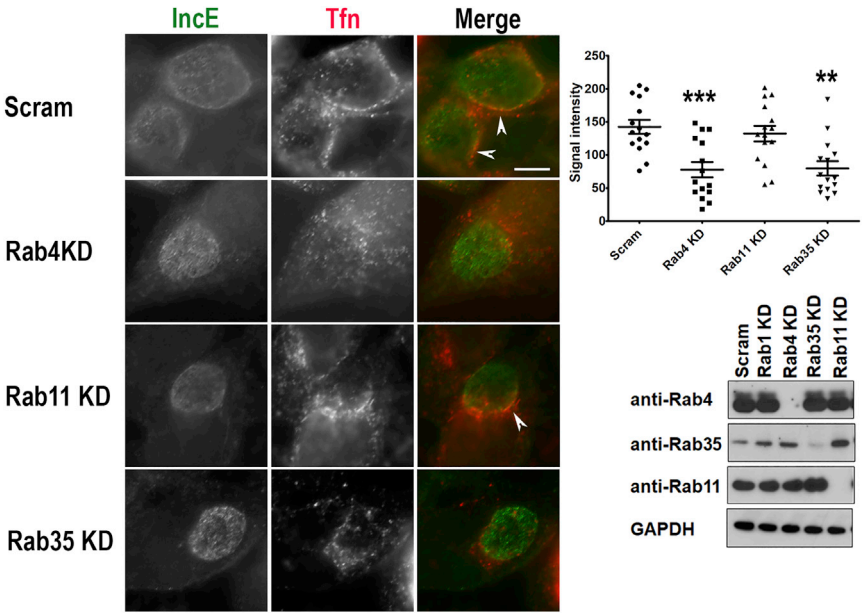


Figure 6. Recruitment of Tfn to the Periphery of the Inclusion Requires Rab4 and 35

Rab KD cells were infected at a MOI of 1 with wild-type *C. trachomatis* L2. At 18 h post-infection, serum-starved infected cells were loaded with Tfn AlexaFluor 594 and after 30 min of recycling, cells were fixed with formaldehyde. Bacteria where stained with anti-IncE (green) antibodies. KD efficiency was determined by western blotting with GAPDH as a loading control. Data are representative of three independent experiments. Scale bars are 10 μm. Arrowheads indicate areas where Tfn is recruited to the inclusion. Fluorescence intensity at the inclusion membrane was quantified from 20 infected cells using ImageJ. Statistical analysis was tabulated using one-way ANOVA with Tukey's as a post-test and generated a statistical difference of $p < 0.001$ (***) or $p < 0.01$ (**).

many metabolic enzymes. As such, the bacteria must scavenge crucial nutrients, such as lipids, iron, and amino acids, from the host cell to proliferate and cause disease (Elwell et al., 2016). To obtain key nutritional substrates from the host, *C. trachomatis* employs CT229 as a keystone effector protein to redirect host vesicular traffic toward the chlamydial inclusion. Through interactions with a plethora of Rab GTPases, CT229 reroutes Rab-containing vesicles to the inclusion. Notably, CT229 redirects Tfn from the recycling pathway and M6PR-containing vesicles to the periphery of the inclusion. Strikingly, the absence of CT229 results in defects in homotypic fusion and premature inclusion lysis, presumably due to its inability to acquire essential substrates for incorporation into the bacterial cell and inclusion membrane (Figure 7).

Several studies have noted the importance of the Inc protein CT229 for productive chlamydial infection; however, the overarching physiological impact of this Inc on host processes during infection has remained elusive. CT229 has been shown to interact with multiple Rab GTPases (Mirrashidi et al., 2015; Rzomp et al., 2006; Sixt et al., 2017), but until now, whether these Rab-containing vesicles are recruited to vicinity of the chlamydial inclusion during infection, and their role in chlamydia infection was unknown. Here, we show that CT229 directly binds and recruits Rab GTPases, perhaps associated with vesicles, that are involved in anterograde transport (Rab1), retrograde transport (Rab6), post-Golgi transport (Rab8, 10, 14), intra-Golgi transport (Rab34), and protein recycling (Rab4, 35). Although CT229 binds and recruits a striking number of Rab GTPases, Rabs, such as Rab5, 7, and 9, are not recruited to the chlamydial inclusion (Rzomp et al., 2003). On the other hand, other Rabs, such as Rab11, are recruited independent of CT229. The selective recruitment or exclusion of specific Rab GTPases allows chlamydiae to tightly control interactions with the host cell, allowing for avoidance of the endocytic/lysosomal pathway while promoting interactions with the recycling pathway. The diversity of Rabs that CT229 binds suggests it may be involved in manipulation of multiple host vesicular trafficking pathways, potentially to acquire key biosynthetic precursors and essential nutrients, such as lipids and iron. Alternatively, CT229 may recruit a plethora of Rab GTPases to camouflage the inclusion as a secretory vesicle or recycling endosome to avoid targeting to the lysosome.

To carry out their effector function, GTP-bound Rab GTPases interact with specific Rab effector proteins. Previous studies have shown that Rab effectors, such as BICD1 and OCRL, localize to the inclusion (Moorhead et al., 2007, 2010). Here, we show that Rab effector proteins, such as OCRL and RUFY1, localize in close proximity to the chlamydial inclusion in a CT229-dependent manner. This suggests that localization of specific Rab effectors proximal to the inclusion is mediated through interactions with their cognate GTPase and that they themselves may not be directly recruited. The association of Rab effectors with Rab GTPases near the inclusion would allow for essential functions, such as vesicle uncoating and tethering to occur and formation of this complex at the surface of the inclusion membrane would allow for more efficient hijacking of CCV trafficking by the bacterium. In contrast to CT229, the T4SS effector protein LidA from *Legionella pneumophila* binds to Rab1 and prevents its inactivation by host GTPase activating proteins (GAPs), thus keeping it in an activate conformation (Neunuebel et al., 2012). Surprisingly, binding of LidA to Rab1 prevents association with Rab effector proteins, which may represent a unique way for *L. pneumophila* to restrict Rab interactions to a specific set of Rab ligands (Neunuebel et al., 2012). We hypothesize that similar to LidA, CT229 interacts with and recruits GTP-bound Rab GTPases to the inclusion and perpetuates signaling by maintaining the Rab GTPase in an active conformation. However, in contrast to LidA, our data suggest that CT229 allows the Rab GTPase to interact with Rab effector proteins. Whether CT229 directly affects the GTP-bound state of the GTPase or simply prevents its inactivation warrants further study.

Bioinformatic analysis revealed a putative coiled-coil SLD within CT229 which we show to be essential for intracellular

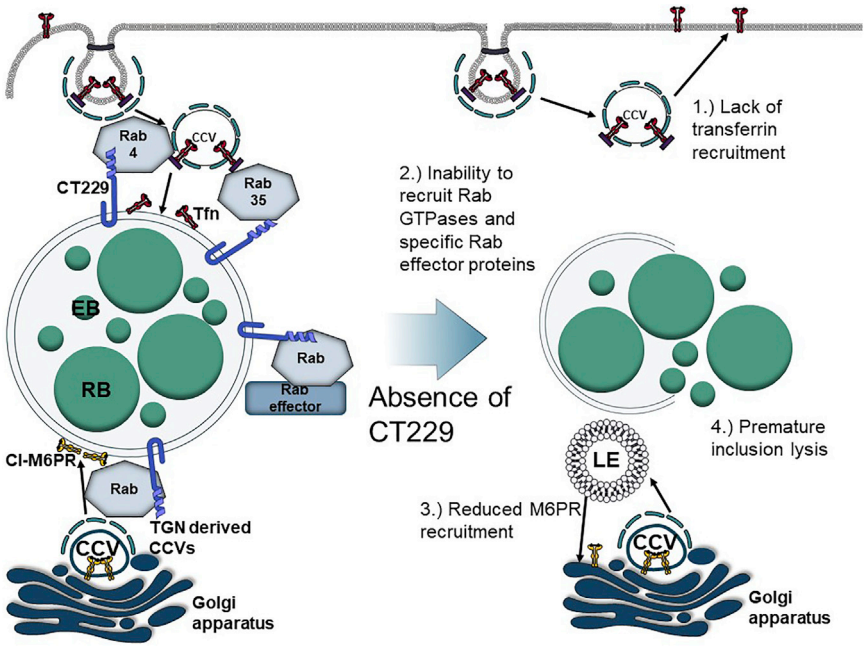


Figure 7. Model for CT229-Dependent Modulation of Host CCV Transport

CT229 interacts with a plethora of Rab GTPases and specifically recruits Rab proteins and their cognate effectors to the inclusion to redirect host Tfn and CI-M6PR toward the inclusion.

replication and recruitment of Rab GTPases to the inclusion. Mutation of a single amino acid residue in the SLD domain ($CT229^{L120D}$) significantly reduced Rab binding and recruitment to the inclusion, suggesting that the SLD of CT229 is essential for interactions with host vesicle trafficking pathways. Eukaryotic SNARE proteins mediate fusion of vesicles with membranes and are not known for their ability to bind and capture Rab GTPases. It is possible that CT229 acts in concert with either a host SNARE or another Inc protein to mediate fusion of vesicles with the inclusion membrane. Multifunctionality of bacterial effector proteins is not uncommon and we speculate that CT229 may both capture and initiate fusion of certain host vesicles with the inclusion membrane.

The CI-M6PR in the TGN recognizes M6P residues on lysosomal proteins and promotes their delivery to prelysosomal compartments, where the drop in pH promotes ligand dissociation and the CI-M6PR is subsequently recycled back to the TGN (Braulke and Bonifacino, 2009). During trafficking, the CI-M6PR associates with the retromer complex through interactions with sorting nexin (SNX)5 and SNX6. Using an affinity purification mass spectrometry (AP-MS) screen, an interaction between IncE and SNX5/6 was detected (Mirrashidi et al., 2015). Structural analysis of the IncE-SNX5 interaction revealed that IncE binds to a conserved hydrophobic groove in the PX domain, the same residues in which the CI-M6PR receptor binds (Elwell et al., 2017; Paul et al., 2017; Sun et al., 2017) The interaction with IncE disrupts CI-M6PR trafficking, which may act to decrease lysosome potency or to access critical nutrients (Elwell and Engel, 2018). Here, we demonstrate that CT229 is required to recruit CI-M6PR-containing vesicles to the vicinity of the inclusion. Whether CT229 interacts in a cooperative manner with IncE to tether vesicles to the inclusion membrane or if this is a compensatory mechanism occurring independently of IncE warrants further study.

Acquisition of iron is typically an infection limiting step during pathogenic bacterial infections (Pokorzynski et al., 2017). As such, chlamydia requires iron to complete its replicative cycle; however, the mechanism(s) by which chlamydia acquires iron from the host cell is unknown. Iron is essential for productive chlamydial infection, and although it has been known for 20 years that transferrin localizes to the periphery of the inclusion membrane, the mechanisms utilized by the bacterium to recruit Tfn have remained elusive (Al-Younes et al., 2001; van Ooij et al., 1997; Scidmore et al., 1996). Although Tfn localizes to the periphery of the inclusion, it is not observed within the inclusion, suggesting that if Tfn represents a source of iron for the bacteria, it is freed either prior to or during fusion with the inclusion membrane. Once inside the inclusion, iron may be transported via unknown siderophores, carriers, or specific ion channels into the bacterial cell. Here, we show that interactions between CT229 and Rab4 or 35 positive vesicles are required for Tfn localization to the periphery of the inclusion. These data potentially provide an important clue about how *C. trachomatis* may subvert iron from the host without disrupting the cellular labile iron pool and homeostasis.

In summary, we show that the essential inclusion membrane protein CT229 is required to bind and redirect numerous Rabs or Rab-containing vesicles to the inclusion. Importantly, our study demonstrates that CT229 and specific Rab GTPases, such as Rab4 and 35, are necessary to redirect Tfn-containing vesicles to the periphery of the inclusion. The observation that specific Rab effectors are recruited to the inclusion in a manner that requires CT229 implies that chlamydiae promote the formation of a complex of Rab GTPases and Rab effector proteins to more efficiently capture host vesicles. By linking Inc function to manipulation of specific Rab GTPases, we have uncovered a potential mechanism used by this important human pathogen to acquire key nutriential substrates from the host.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Chlamydia propagation
  - Cell Lines

SUPPLEMENTAL INFORMATION

Supplemental Information can be found with this article online at https://doi.org/10.1016/j.celrep.2019.02.079.

ACKNOWLEDGMENTS

This work was supported by startup funds from the University of Iowa to M.M.W. We thank Fabienne Paumet for assistance with bioinformatics. We also thank Janet Sager and Tina Clark for technical assistance.

AUTHOR CONTRIBUTIONS

M.M.W., R.F., and T.H. conceived of the experiments. M.M.W., R.F., M.M., S.E.A., and C.A.D performed the experiments. M.M.W. and R.F. analyzed the data. M.M.W. and R.F. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: August 30, 2018
Revised: November 28, 2018
Accepted: February 20, 2019
Published: March 19, 2019

REFERENCES

Afrakhteh, M., Mahdavi, A., Beyhaghi, H., Moradi, A., Gity, S., Zafargandi, S., and Zonoubi, Z. (2013). The prevalence of *Chlamydia trachomatis* in patients who remained symptomatic after completion of sexually transmitted infection treatment. Iran. J. Reprod. Med. *11*, 285–292.

Al-Younes, H.M., Rudel, T., Brinkmann, V., Szczepek, A.J., and Meyer, T.F. (2001). Low iron availability modulates the course of *Chlamydia pneumoniae* infection. Cell. Microbiol. *3*, 427–437.

Braulke, T., and Bonifacino, J.S. (2009). Sorting of lysosomal proteins. Biochim. Biophys. Acta *1793*, 605–614.

Caldwell, H.D., Kromhout, J., and Schachter, J. (1981). Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. *31*, 1161–1176.

Carabeo, R.A., Mead, D.J., and Hackstadt, T. (2003). Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion. Proc. Natl. Acad. Sci. USA *100*, 6771–6776.

Cauvin, C., Rosendale, M., Gupta-Rossi, N., Rocancourt, M., Larraufie, P., Salomon, R., Perrais, D., and Echard, A. (2016). Rab35 GTPase triggers switch-like recruitment of the Lowe syndrome lipid phosphatase OCRL on newborn endosomes. Curr. Biol. *26*, 120–128.

Chowdhury, S.R., Reimer, A., Sharan, M., Kozjak-Pavlovic, V., Eulalio, A., Prusty, B.K., Fraunholz, M., Karunakaran, K., and Rudel, T. (2017). *Chlamydia* preserves the mitochondrial network necessary for replication via microRNA-dependent inhibition of fission. J. Cell Biol. *216*, 1071–1089.

Delevoye, C., Nilges, M., Dehoux, P., Paumet, F., Perrinet, S., Dautry-Varsat, A., and Subtil, A. (2008). SNARE protein mimicry by an intracellular bacterium. PLoS Pathog. *4*, e1000022.

Derré, I., Swiss, R., and Agaisse, H. (2011). The lipid transfer protein CERT interacts with the Chlamydia inclusion protein IncD and participates to ER-Chlamydia inclusion membrane contact sites. PLoS Pathog. *7*, e1002092.

Donaldson, J.G., and Jackson, C.L. (2011). ARF family G proteins and their regulators: roles in membrane transport, development and disease. Nat. Rev. Mol. Cell Biol. *12*, 362–375.

Elwell, C., and Engel, J. (2018). Emerging role of retromer in modulating pathogen growth. Trends Microbiol. *26*, 769–780.

Elwell, C.A., Czudnochowski, N., von Dollen, J., Johnson, J.R., Nakagawa, R., Mirrashidi, K., Krogan, N.J., Engel, J.N., and Rosenberg, O.S. (2017). *Chlamydia* interfere with an interaction between the mannose-6-phosphate receptor and sorting nexins to counteract host restriction. eLife *6*, 1–17.

Elwell, C.A., Mirrashidi, K., and Engel, J. (2016). Chlamydia cell biology and pathogenesis. Nat. Rev. Microbiol. *14*, 385–400.

Finethy, R., and Coers, J. (2016). Sensing the enemy, containing the threat: cell-autonomous immunity to *Chlamydia trachomatis*. FEMS Microbiol. Rev. *40*, 875–893.

Geisler, W.M., Uniyal, A., Lee, J.Y., Lensing, S.Y., Johnson, S., Perry, R.C.W., Kadrnka, C.M., and Kerndt, P.R. (2015). Azithromycin versus Doxycycline for urogenital *Chlamydia trachomatis* infection. N. Engl. J. Med. *373*, 2512–2521.

Grieshaber, S.S., Grieshaber, N.A., and Hackstadt, T. (2003). *Chlamydia trachomatis* uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamitin-independent process. J. Cell Sci. *116*, 3793–3802.

Guo, Z., Stephenson, R., Qiu, J., Zheng, S., and Luo, Z.Q. (2014). A Legionella effector modulates host cytoskeletal structure by inhibiting actin polymerization. Microbes Infect. *16*, 225–236.

Hackstadt, T., Scidmore-Carlson, M.A., Shaw, E.I., and Fischer, E.R. (1999). The *Chlamydia trachomatis* IncA protein is required for homotypic vesicle fusion. Cell. Microbiol. *1*, 119–130.

Hammer, J.A., 3rd, and Wu, X.S. (2002). Rabs grab motors: defining the connections between Rab GTPases and motor proteins. Curr. Opin. Cell Biol. *14*, 69–75.

Heuer, D., Rejman Lipinski, A., Machuy, N., Karlas, A., Wehrens, A., Siedler, F., Brinkmann, V., and Meyer, T.F. (2009). Chlamydia causes fragmentation of the Golgi compartment to ensure reproduction. Nature *457*, 731–735.

Hutagalung, A.H., and Novick, P.J. (2011). Role of Rab GTPases in membrane traffic and cell physiology. Physiol. Rev. *91*, 119–149.

Jennings, E., Thurston, T.L.M., and Holden, D.W. (2017). Salmonella SPI-2 type III secretion system effectors : molecular mechanisms and physiological consequences. Cell Host Microbe *22*, 217–231.

Kim, K., and Gadila, S.K.G. (2016). Cargo trafficking from the trans-Golgi network towards the endosome. Biol. Cell *108*, 205–218.

Mayle, K.M., Le, A.M., and Kamei, D.T. (2012). The intracellular trafficking pathway of transferrin. Biochim. Biophys. Acta *1820*, 264–281.

Menon, S., Timms, P., Allan, J.A., Alexander, K., Rombauts, L., Horner, P., Keltz, M., Hocking, J., and Huston, W.M. (2015). Human and pathogen factors associated with *Chlamydia trachomatis*-related infertility in women. Clin. Microbiol. Rev. *28*, 969–985.

Mirrashidi, K.M., Elwell, C.A., Verschueren, E., Johnson, J.R., Frando, A., Von Dollen, J., Rosenberg, O., Gulbahce, N., Jang, G., Johnson, T., et al. (2015). Global mapping of the inc-human interactome reveals that retromer restricts chlamydia infection. Cell Host Microbe *18*, 109–121.

Moorhead, A.R., Rzomp, K.A., and Scidmore, M.A. (2007). The Rab6 effector Bicaudal D1 associates with *Chlamydia trachomatis* inclusions in a biovar-specific manner. Infect. Immun. *75*, 781–791.

Moorhead, A.M., Jung, J.Y., Smirnov, A., Kaufer, S., and Scidmore, M.A. (2010). Multiple host proteins that function in phosphatidylinositol-4-phosphate metabolism are recruited to the chlamydial inclusion. Infect. Immun. *78*, 1990–2007.

Muschiol, S., Boncompain, G., Vromman, F., Dehoux, P., Normark, S., Henriques-Normark, B., and Subtil, A. (2011). Identification of a family of effectors secreted by the type III secretion system that are conserved in pathogenic Chlamydiae. Infect. Immun. *79*, 571–580.

Nández, R., Balkin, D.M., Messa, M., Liang, L., Paradise, S., Czapla, H., Hein, M.Y., Duncan, J.S., Mann, M., and De Camilli, P. (2014). A role of OCRL in clathrin-coated pit dynamics and uncoating revealed by studies of Lowe syndrome cells. eLife *3*, e02975.

Neunuebel, M.R., Mohammadi, S., Jarnik, M., and Machner, M.P. (2012). *Legionella pneumophila* LidA affects nucleotide binding and activity of the host GTPase Rab1. J. Bacteriol. *194*, 1389–1400.

Patel, A.L., Chen, X., Wood, S.T., Stuart, E.S., Arcaro, K.F., Molina, D.P., Petrovic, S., Furdui, C.M., and Tsang, A.W. (2014). Activation of epidermal growth factor receptor is required for *Chlamydia trachomatis* development. BMC Microbiol. *14*, 277.

Paul, B., Kim, H.S., Kerr, M.C., Huston, W.M., Teasdale, R.D., and Collins, B.M. (2017). Structural basis for the hijacking of endosomal sorting nexin proteins by *Chlamydia trachomatis*. eLife *6*, 1–23.

Pokorzynski, N.D., Thompson, C.C., and Carabeo, R.A. (2017). Ironing out the unconventional mechanisms of iron acquisition and gene regulation in chlamydia. Front. Cell. Infect. Microbiol. *7*, 394.

Progida, C., and Bakke, O. (2016). Bidirectional traffic between the Golgi and the endosomes - machineries and regulation. J. Cell Sci. *129*, 3971–3982.

Qiu, J., and Luo, Z.Q. (2017). Legionella and Coxiella effectors: strength in diversity and activity. Nat. Rev. Microbiol. *15*, 591–605.

Robinson, M.S. (2015). Forty Years of Clathrin-coated Vesicles. Traffic *16*, 1210–1238.

Rzomp, K.A., Scholtes, L.D., Briggs, B.J., Whittaker, G.R., and Scidmore, M.A. (2003). Rab GTPases are recruited to chlamydial inclusions in both a species-dependent and species-independent manner. Infect. Immun. *71*, 5855–5870.

Rzomp, K.A., Moorhead, A.R., and Scidmore, M.A. (2006). The GTPase Rab4 interacts with *Chlamydia trachomatis* inclusion membrane protein CT229. Infect. Immun. *74*, 5362–5373.

Saka, H.A., Thompson, J.W., Chen, Y.S., Dubois, L.G., Haas, J.T., Moseley, A., and Valdivia, R.H. (2015). *Chlamydia trachomatis* infection leads to defined alterations to the lipid droplet proteome in epithelial cells. PLoS ONE *10*, e0124630.

Satpathy, G., Behera, H.S., and Ahmed, N.H. (2017). Chlamydial eye infections: current perspectives. Indian J. Ophthalmol. *65*, 97–102.

Scidmore, M.A., Fischer, E.R., and Hackstadt, T. (1996). Sphingolipids and glycoproteins are differentially trafficked to the *Chlamydia trachomatis inclusion*. J. Cell Biol. *134*, 363–374.

Scidmore, M.A., Fischer, E.R., and Hackstadt, T. (2003). Restricted fusion of *Chlamydia trachomatis* vesicles with endocytic compartments during the initial stages of infection. Infect. Immun. *71*, 973–984.

Scidmore-Carlson, M.A., Shaw, E.I., Dooley, C.A., Fischer, E.R., and Hackstadt, T. (1999). Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. Mol. Microbiol. *33*, 753–765.

Sherwood, R.K., and Roy, C.R. (2013). A Rab-centric perspective of bacterial pathogen-occupied vacuoles. Cell Host Microbe *14*, 256–268.

Sixt, B.S., Bastidas, R.J., Finethy, R., Baxter, R.M., Carpenter, V.K., Kroemer, G., Coers, J., and Valdivia, R.H. (2017). The *Chlamydia trachomatis* inclusion membrane protein CpoS counteracts STING-mediated cellular surveillance and suicide programs. Cell Host Microbe *21*, 113–121.

Stoner, B.P., and Cohen, S.E. (2015). Lymphogranuloma Venereum 2015: Clinical presentation, diagnosis, and treatment. Clin. Infect. Dis. *61* (*Suppl 8*), S865–S873.

Subtil, A., Delevoye, C., Balañá, M.E., Tastevin, L., Perrinet, S., and Dautry-Varsat, A. (2005). A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. Mol. Microbiol. *56*, 1636–1647.

Sun, Q., Yong, X., Sun, X., Yang, F., Dai, Z., Gong, Y., Zhou, L., Zhang, X., Niu, D., Dai, L., et al. (2017). Structural and functional insights into sorting nexin 5/6 interaction with bacterial effector IncE. Signal Transduct. Target. Ther. *2*, 17030.

Tan, Y., and Luo, Z.Q. (2011). *Legionella pneumophila* SidD is a deAMPylase that modifies Rab1. Nature *475*, 506–509.

Tan, Y., Arnold, R.J., and Luo, Z.-Q. (2011). *Legionella pneumophila* regulates the small GTPase Rab1 activity by reversible phosphorylcholination. Proc. Natl. Acad. Sci. USA *108*, 21212–21217.

van Ooij, C., Apodaca, G., and Engel, J. (1997). Characterization of the *Chlamydia trachomatis* vacuole and its interaction with the host endocytic pathway in HeLa cells. Infect. Immun. *65*, 758–766.

Weber, M.M., and Faris, R. (2018). Subversion of the endocytic and secretory pathways by bacterial effector proteins. Front. Cell Dev. Biol. *6*, 1–17.

Weber, M.M., Chen, C., Rowin, K., Mertens, K., Galvan, G., Zhi, H., Dealing, C.M., Roman, V.A., Banga, S., Tan, Y., et al. (2013). Identification of *Coxiella burnetii* type IV secretion substrates required for intracellular replication and Coxiella-containing vacuole formation. J. Bacteriol. *195*, 3914–3924.

Weber, M.M., Bauler, L.D., Lam, J., and Hackstadt, T. (2015). Expression and localization of predicted inclusion membrane proteins in *Chlamydia trachomatis*. Infect. Immun. *83*, 4710–4718.

Weber, M.M., Faris, R., van Schaik, E.J., McLachlan, J.T., Wright, W.U., Tellez, A., Roman, V.A., Rowin, K., Case, E.D., Luo, Z.-Q., and Samuel, J.E. (2016a). The type IV secreted effector protein CirA stimulates the GTPase activity of RhoA and is required for virulence in a mouse model of *Coxiella burnetii* infection. Infect. Immun. *84*, 2524–2533.

Weber, M.M., Noriea, N.F., Bauler, L.D., Lam, J.L., Sager, J., Wesolowski, J., Paumet, F., and Hackstadt, T. (2016b). A functional core of IncA is required for *Chlamydia trachomatis* inclusion fusion. J. Bacteriol. *198*, 1347–1355.

Weber, M.M., Lam, J.L., Dooley, C.A., Noriea, N.F., Hansen, B.T., Hoyt, F.H., Carmody, A.B., Sturdevant, G.L., and Hackstadt, T. (2017). Absence of specific *Chlamydia trachomatis* inclusion membrane proteins triggers premature inclusion membrane lysis and host cell death. Cell Rep. *19*, 1406–1417.

Yamamoto, H., Koga, H., Katoh, Y., Takahashi, S., Nakayama, K., and Shin, H.-W. (2010). Functional cross-talk between Rab14 and Rab4 through a dual effector, RUFY1/Rabip4. Mol. Biol. Cell *21*, 2746–2755.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit-anti-GFP	AbCam	Ab6556
DYKDDDDK Tag Rabbit Oligoclonal	ThermoFisher	710662
Rabbit-anti-Rab8	AbCam	Ab65200
Rabbit-anti-Rab11	AbCam	Ab188574
Rabbit-anti-Rab35	AbCam	Ab152138
Rabbit-anti-Rab4	Cell Signaling	2167
Transferrin Alexa 594 conjugate	ThermoFisher	T13343
EGF Tetramethylrhodamine conjugate	ThermoFisher	E3481
CI-M6PR	ThermoFisher	PA3-850
Monoclonal Anti-HA antibody produced in mouse	Sigma	H9658
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	ThermoFisher	A-11012
F(ab')2-Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	ThermoFisher	A-21205
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	R37116
Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	A-11059
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate	BioRad	1706515
Rabbit-anti-IncE	Ted Hackstadt	NA
Mouse-anti-momp	Ted Hackstadt	NA
Rabbit-anti-L2	Ted Hackstadt	NA
Transferrin HRP	ThermoFisher	31453
Bacterial and Virus Strains		
Chlamydia trachomatis L2	Ted Hackstadt	NA
Chemicals, Peptides, and Recombinant Proteins		
Renografin (MD-76R)	Mallinckrodt	00019131707
DAPI	ThermoFisher	D1306
Opti-MEM medium	ThermoFisher	31985062
Lipofectamine	ThermoFisher	15338100
RPMI 1640 medium with L-glutamine	ThermoFisher	11875-093
Sodium bicarbonate	ThermoFisher	25080094
Sodium pyruvate	ThermoFisher	11360070
Gentamicin	ThermoFisher	15710072
Live cell imagine solution	ThermoFisher	A14291DJ
Trypsin	ThermoFisher	15400054
Fetal Bovine Serum	VWR	89510-186
PBS	ThermoFisher	10010-049
SacII	NEB	R0157S
SalI	NEB	R3138S
EcoRI	NEB	R3101S
XhoI	NEB	R0146S
Dharmafect	Dharmacon	T-2001-01
Formaldehyde	ThermoFisher	28908

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-Flag M2 affinity resin	Millipore sigma	A2220
Rab4 siRNA	Dharmacon	L-008539-00-0020 20 nmol
Rab11 siRNA	Dharmacon	L-004726-00-0020 20 nmol
Rab35 siRNA	Dharmacon	L-009781-00-0020 20 nmol
ON-TARGETplus Non-targeting Pool	Dharmacon	D-001810-10-20
ImmunoPure Metal Enhanced DAB Substrate	ThermoFisher	34065
Yeast Nitrogen base	Sigma	Y0626-250G
Uracil dropout supplement	Sigma	Y1501-20G
Uracil leucine dropout supplement	Sigma	Y1771-20G
Sucrose	Fisher scientific	BP220-1
Glucose	Fisher scientific	D16-500
Galactose	Fisher scientific	BP656-500
Sodium phosphate, dibasic	Fisher scientific	S374-500
Sodium phosphate, monobasic	Fisher scientific	S369-500
Glutamic Acid	Fisher scientific	A125-100
Ultrapure low melting point agarose	ThermoFisher	***16520050***
Ultrapure agarose	ThermoFisher	***16500500***
Ethidium bromide solution	ThermoFisher	***17896***
ProLong Gold Antifade Mountant	ThermoFisher	***P36934***
Triton X-100	Fisher scientific	***BP151-100***
Bovine Serum Albumin	Fisher scientific	***BP1605-100***
Anhydrotetracycline	Sigma	***37919-100MG-R***
Tris HCl	Fisher scientific	***PR-H5121***
Sodium chloride	Fisher scientific	***S271-500***
Ethylenediaminetetraacetic Acid	Fisher scientific	***S311-100***
NuPAGE 4–12% Bis-Tris gels	ThermoFisher	***NP0336BOX***
NuPAGE MES SDS Running Buffer	ThermoFisher	***NP0002***
NuPAGE LDS Sample Buffer (4X)	ThermoFisher	***NP0007***
PVDF Transfer Membrane, 0.45 μm, 10 cm x 10 cm	ThermoFisher	***88585***
Methanol	Fisher Scientific	***A412-500***
Nunc Thermanox coverslips	ThermoFisher	***174950***
Glutaraldehyde	Sigma	***G5882-10X10ML***
Experimental Models: Cell Lines		
HeLa cells	ATCC	CCL-2
Vero cells	ATCC	CCL81
Oligonucleotides		
CT229 EcoRI F	IDT	CCGAATTCATGAGCTGTTCTAATGTTAATTCAGGT
CT229 XhoI R	IDT	CCCTCGACTTTTTTACGACGGGATGCC
CT229 Npro SacII F	IDT	CCCCGCGGACCAAGCAGTATGCTTAAGCCA
CT229 Flag SalI R	IDT	CCGTCGACttacttatcgtcgtcatccttgtaatcTTTTTTACGAC GGGATGCC
CT229 NotI F	IDT	CCGCGGCCGCATGAGCTGTTCTAATGTTAATTCAGGT
pYep13 F	IDT	ACTACGCGATCATGGCGA
pYep13 R	IDT	TGATGCCGGCCACGATGC
Recombinant DNA		
CT229 L120D	GenScript	NA
pcDNA	GenScript	NA
EGFP-Rab1A	Addgene	49467

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
EGFP-Rab1A Q70L	Addgene	49537
EGFP-Rab1A S25N	Addgene	49539
EGFP-Rab4A	Addgene	49434
EGFP-Rab4A Q67L	Addgene	49475
EGFP-Rab4A S22N	Addgene	49476
EGFP-Rab35	Addgene	49552
EGFP-Rab35 Q67L	Addgene	49612
EGFP-Rab35 S22N	Addgene	49613
EGFP-Rab8	Addgene	86075
pcDNA GFP Rab6	GenScript	OHu17943
pcDNA GFP Rab11	GenScript	OHu15335
pcDNA GFP Rab10	GenScript	OHu12758
pcDNA GFP Rab14	GenScript	OHu12700
pcDNA GFP Rab18	GenScript	OHu22752
pcDNA GFP Rab33	GenScript	OHu29415
pcDNA GFP Rab34	GenScript	OHu26377
pcDNA RUFY1	GenScript	OHu55831
pcDNA HA OCRL	Addgene	22207
pYes2NTA	Life Technologies	V82520
pYep13	ATCC	37323
Software and Algorithms		
GraphPad Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
ImageJ	NIH	https://imagej.nih.gov/ij/
ELM	NA	http://elm.eu.org/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mary M. Weber (mary-weber@uiowa.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Chlamydia propagation

Chlamydia trachomatis serovar L2 (LGV 434/Bu) was propagated in HeLa 229 cells (American Type Culture Collection) grown in T150 flasks in 30ml RPMI 1640 with L-glutamine (ThermoFisher Cat#11875-093) supplemented with 10% Fetal Bovine Serum (FBS) (VWR Cat#89510-186) and 1mM sodium pyruvate (ThermoFisher Cat#11360070). At 48hr post-infection, EBs were purified using a Renografin density gradient (Caldwell et al., 1981). Purified EBs were stored in SPG in 50μl aliquots at −80°C.

Cell Lines

HeLa (isolated from a female) and Vero CCL-81 cells (isolated from a female) (ATCC) were propagated in RPMI 1640 with L-glutamine and supplemented with 10% FBS and 1mM sodium pyruvate. Cells were grown at 37°C in a humidified incubator with 5% CO_2.

METHOD DETAILS

Complementation of CT229::bla

CT229 and 300 base pairs upstream were PCR amplified and cloned into the SacII/SalI site of pBomb3 (Weber et al., 2016b). Site-specific mutagenesis to generate CT229 L120D was performed by GenScript. *C. trachomatis* CT229::*bla* was transformed as previously described and transformats were purified by plaque cloning (Weber et al., 2016b). Expression of the fusion protein was verified by IF microscopy.

Growth curve analysis

HeLa cells were infected at an MOI of 1 and at 0, 24, and 48 h post-infection cells were lysed in water, serially diluted, and supernatants were applied to fresh HeLa monolayers in 24 well plates. Infected cells were fixed with methanol and stained with an anti-*C. trachomatis* L2 antibody. Inclusions were counted by IF microscopy.

Immunofluorescence

HeLa cells were seeded onto glass coverslips and transfected with Rab GTPases using Lipofectamine LTX as previously described (Weber et al., 2013). Four hr post-transfection, HeLa cells were infected at an MOI of 1. Eighteen hr post-infection, cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked using 1% BSA. The inclusion membrane was stained using an anti-IncE antibody (1:500) or bacteria were stained using anti-MOMP (1:500). Endogenous Rab GTPases were detected using antibodies purchased through abcam. Epifluorescence images were captured on a Nikon TI2 scope and fluorescence intensity at the inclusion membrane was quantified from 20 infected cells using ImageJ. Data are representative of at least 3 independent experiments with at least 100 infected cells per experiment.

Immunoprecipitation

GFP-tagged Rab GTPases and Rab effectors were purchased from AddGene or GenScript and used to transfect HeLa cells. Four hr post-transfection, HeLa cells were infected at an MOI of 2.5 with *C. trachomatis* L2 pBomb4-tet-CT229 or -CT224 Flag (Weber et al., 2015) and expression of the Flag-tagged fusion was induced with 10ng aTc. Twenty-four hr post-infection, infected cells were lysed using lysis buffer (50mM Tris HCl, pH7.4, 150mM NaCl, 1mM EDTA, and 1% Triton X-100) and flag-tagged fusion protein was immunoprecipitated using anti-Flag M2 affinity agarose (Sigma-Aldrich). Samples were resolved using a NuPAGE 4%–12% Bis-Tris protein gel and were subsequently transferred to a PVDF membrane. Membranes were probed with anti-Flag (1:5000) (ThermoFisher) or anti-GFP antibodies (1:5000) (Abcam). Data are representative of at least 3 independent experiments.

siRNA knockdown of host genes

siRNA transfections were performed on HeLa cells seeded on 24-well plates as previously described (Weber et al., 2017). After 24 h, cells were transfected with siRNA to the transcript of interest or On-Target Control siRNA using Dharmafect 1 (Dharmacon) and plates were incubated for 48h at 37°C. At 48h, HeLa cells were infected at an MOI of 1 and incubated for an addition 18h. Infectious EBs were enumerated by lysing host cells in water, serially diluting the lysate, and applying the supernatants to a fresh HeLa monolayer in 24 well plates. Infected cells were fixed with methanol and stained with an anti-*C. trachomatis* L2 antibody (1:1000). Inclusions were counted by IF microscopy. Ten fields in triplicate were counted per experiment. Rab knockdown was confirmed by western blotting.

Yeast suppressor screen

CT229 was cloned into the EcoRI/XhoI site of pYesNTA2 and growth was assessed by serial dilution and spotting on uracil dropout medium containing galactose. The pYEp13 genomic library (ATCC no. 37323) was transformed into *Saccharomyces* cerevisiae W303 (pGal::CT229) and the resulting transformants were plated on uracil leucine dropout medium containing galactose as previously described (Weber et al., 2016a). To verify suppression, plasmids were isolated from the rescues and retransformed into *S. cerevisiae* W303 (pGal::CT229). Suppressor plasmids were sequenced using pYEp13 seq F (ACTACGCGATCATGGCGA) and pYEp13 seq R (TGATGCCGGCCACGATGC) and sequences were analyzed using the yeast genome database (https://www.yeastgenome.org/) to identify the yeast *orfs*.

Recycling assays

HeLa cells were seeded onto glass coverslips and infected at an MOI of 1. At 18 hr post-infection the media was replaced with Live Cell Imaging Solution (LCIS) (ThermoFisher Scientific) and cells were placed on ice for 20min. Infected cells were loaded with Tfn AlexaFluor 594 conjugate (25μg/ml in LCIS) or EGF Tetramethylrhodamine conjugate (10μg/ml in LCIS) and incubated on ice for 20min. Cells were washed twice with ice-cold LCIS and loaded with excess unlabeled Tfn or EGF. Samples were incubated at 37°C for 0, 30, or 60min. Cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked using 1% BSA. *C. trachomatis* was stained with an anti-L2 antibody (1:1000) or anti-IncE (1:500). Data are representative of at least 3 independent experiments with at least 100 infected cells per experiment.

Immunoelectron microscopy

HeLa cells were seeded on Thermanox coverslips and infected at an MOI of 2 for 18 h. Coverslips were fixed in periodate-lysine-paraformaldehyde (PLP) with 0.25% glutaraldehyde for 2 h at room temperature. Cells were subsequently incubated in the presence of Tfn-HRP as previously described (Scidmore et al., 1996). Samples were fixed in 2% glutaraldehyde and incubated with ImmunoPure Metal Enhanced DAB Substrate. After fixation, cells were mixed with OsO_4/1%$K_3Fe(CN)_6$, embedded in Spurr's epoxy resin, and thin sections were cut with an RMC MT-7000 ultramicrotome (Research and Manufacturing Company). Cells were subsequently stained with 1% uranyl acetate and Reynold's lead citrate. Images were captured at 60 kV on a Philips CM-10 transmission electron microscope (FEI).

QUANTIFICATION AND STATISTICAL ANALYSIS

Microscopy quantification

Fluorescence intensity at the inclusion membrane was quantified from 20 infected cells using ImageJ. Data are representative of at least 3 independent experiments with at least 100 infected cells per experiment.

Statistics

When required, statistical analysis was conducted using GraphPad Prism software. One-way ANOVA generated a minimum threshold of significance of using One-Way ANOVA and generated a statistical difference of $p < 0.001$ (***), $p < 0.01$ (**), or $p < 0.05$ (*). Tukey or Dunnett was used as a post-test.